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EFFECT OF NEW BIOLOGICALLY ACTIVE POLYPEPTIDES ON DIHEXADECYL PHOSPHATE VESICLES [1]

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SUMMARY . Effect of new biologically active polypeptides on hexadecyl phosphate vesicles. - The effect that monomeric and trimeric chemotactic peptides may have on the permeation of DHP vesicles was studied by means of electronic absorption spectroscopy. The results show that attaching covalently two or more of the same chemotactic peptides to a suitable carrier molecule produced changes in the permeation of DHP vesicles that deviate from simple additivity rule. Thus a parallelism between the biological potency of the peptides and DHP permeation exists.

Dihexadecylphosphate, vesicles, chemotactic peptides, permeation.

[1] Abbreviations : DHP = dihexadecyl phosphate, $Ru(bpy)_3^{2+}$ = tris(2,2'-bipyridyl) Ruthenium (II) chloride hexahydrate, f-MLP = N-formyl L-methionyl-L-Leucyl-L-phenylalanine, tBoc = terbutoxycarbonyl, DMF = N,N-dimethylformamide

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In the past decade the functional properties of membrane assemblies were the object of investigation by scientists belonging to the physical, chemical or biological community. These researches were triggered by the important role that plasma membranes played as a molecular barrier in biological cells.

Single compartment vesicles can be considered as a model for the study of membrane permeability. Indeed, like real membranes they are made from bilayers. In this work, single compartment vesicles made from DHP were used as a membrane model to ascertain a new concept in pharmacology. This concept based on the finding that attaching covalently two or more of the same pharmacophores (biologically active molecules) to a suitable carrier molecule may produce synergetic biological effects (KRAUS. 1984 and papers mentioned therein) . Indeed it has been pointed out that polypharmacophores may interact with the receptors located on the cell surface causing them to cluster (STAHL. 1978 ; KAWASAKI. 1978 ; KORNFELD. 1976 ; LEE. 1978 ; DINTZIS. 1982 ; TOKES. 1982 ; POMPIPOM. 1981) . This clustering effect of membrane receptors may induce changes in the chemico-physical properties of the membrane, among them permeability. The marker $Ru(bpy)_3^{2+}$ was encapsulated in DHP vesicles. The permeation of DHP vesicles by various monomeric and polymeric peptides (scheme I) derived from the well-known chemotactic peptide f-MLP (SHOWELL. 1976) was studied by monitoring $Ru(bpy)_3^{2+}$ leakage across the bilayer using visible absorption spectroscopy.

MATERIALS AND METHODS

The unitary monomeric peptide analogs f-MetLeuPheOCH$_3$ 6 and BOCPheLeuPheOCH$_3$ 7 were obtained from Armand Frappier Corporation (Mon-

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treal) Canada. L-Lys-L-Lys, 2 HCl 5 was purchased from Sigma. N,N'-bis (2-hydroxyethyl)-4,13 diaza-18 crown-6 3 been prepared according to Gatto and Gokel procedure (1984) . The coupling with N-tBOC MetLeuPheOH was accomplished using dicyclohexylcarbodiimide in DMF in the presence of 1-hydroxybenzotriazole as catalyst (KONIG. 1970) . Direct deblocking and formylation of the terminal methionine residue was carried out as recently described (LAJOIE. 1984) leading to compound 1. Compound 2 was obtained by coupling 3 with N-tBOCPheLeuPheOH . Condensation of the p-nitrophenylester of N-tBOCMetLeuPheOH with the L-Lysyl-L-Lysyl trifluoroacetate salt in DMF gave a high yield of the corresponding N-tBOC protected intermediate. The latter was deblocked and formylated as described above, leading to compound 4.

All products thus obtained were chromatographically pure as judged by thin layer chromatography and their structures were established by IR, NMR (200 MHz) and mass spectrometry. Satisfactory elemental analyses were obtained in all cases.

Biological activity of these peptides analogs was evaluated using the release of lysozyme from human neutrophils as the assay method (SHOWELL. 1976) . Reproducible results were readily obtained using cells from healthy blood donors. The lysosomal enzyme inducing activity of each peptide derivative was computed from dose-response curves to give the ED50 values (the concentration of compound causing 50% of the maximal release of lysozyme).

DHP vesicles were prepared by sonication with an ultrasonics cell Disruptor filled with a microtip set at 35 W of output power. Typically, 27 mg of DHP was first heated to 80°, then 33 ml of distilled water pre-heated

at 80°C were added, giving a DHP concentration 1.5 10^{-3} mol.l^{-1}. After initial sonication 0.1 mol.l^{-1} NaOH was injected to give an NaOH concentration of 7.5 10^{-4} mol.l^{-1}. For entrapment purposes appropriate volume of $Ru(bpy)_3^{2+}$ were injected after a few minutes of initial sonication (TRICOT. 1983). Sonication was then continued until a constant turbidity was reached. Traces of titanium released by the microtip during sonication were removed by centrifugation. Externally adsorbed cations, those entrapped during sonication but able to readily permeate the vesicle bilayer, and those in the outer bulk solution, could be removed by passing the vesicle dispersion through a column of Bio-Rad AG 50W-X2 cation exchange resin (100-200 mesh, hydrogen form).

Levels of entrapment were determined at room temperature from the residual concentration of $Ru(bpy)_3^{2+}$ measured after treatment with the cation exchange resin. Concentrations of $Ru(bpy)_3^{2}$ were determined spectrophotometrically at 454 nm (TRICOT et al. 1984). The absorbance/scattering spectrum of the vesicle dispersion in the absence of $Ru(bpy)_3^{2+}$ was determined for each sample and corrections made when determining concentrations of $Ru(bpy)_3^{2+}$ from spectrometric measurements. At 1.5 10^{-3} mol.l^{-1} DHP, as used in entrapment experiments, no destabilization of vesicles due to pH changes during resin treatment was observed. (TRICOT, FURLONG, MAU and SADE. 1985).

Before further use of the vesicles with entrapped ions, HCl resulting from the cation exchange resin was removed by ultrafiltration .

Permeability values are reported as PD50's values. PD50 is the required amount of pharmacophores needed to induce at 50% the permeability of DHP vesicles to $Ru(bpy)_3^{2+}$.

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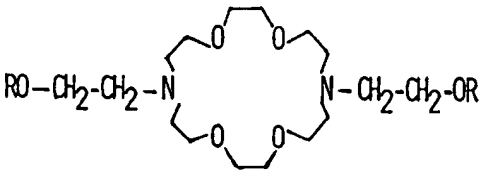
RESULTS AND DISCUSSION

The dimeric and trimeric peptides analogs in Scheme I were synthesized with a view to testing a possible relation between the potency of these compounds to induce lysosomal enzyme release and their effect on the permeation of DHP vesicles. The effect that monomeric analogs 6, 7 and the backbone 3, 5 may have on the permeation of DHP vesicles was also assessed.

The calculated PD50 of FMLP was found to be 4.5×10^{-3} M.L^{-1}. The PD50's of the dimeric 1 peptide and that of the trimeric peptide 4 were found to be 6.3×10^{-5} M.L^{-1} and 6.0×10^{-5} M.L^{-1} respectively. This clearly shows that the trimeric and the dimeric species permeate DHP vesicles by orders to magnitude more than the monomeric species. The PD50's of the carrier molecules were also calculated . The PD50 of Lys.Lys, 2 HCl 5 was found to be 4.15×10^{-4} M.L^{-1}, and 8.10×10^{-4} M.L^{-1} for compound 3. Thus the carrier molecule by itself has a noticeable effect. This can be understood at least for 3, which can be considered as an ionophore. However this effect alone cannot explain the large difference between on the one hand the PD50 of the monomeric species and on the other hand those of the dimeric and trimeric species. Therefore the observed synergetic induced permeation is in part due to the carrier molecules and in part due to the covalent attachment of two or three FMLP entities to those carrier molecules. The results obtained are in line with the observed biological effects (induction of lysosomal enzyme release from human neutrophils). Table I regroups the PD50's of the different studied species as well as their ED50's. Two more compounds known as lysosomes release antagonists (DAY . 1980) were also studied BOC-Phe-Leu-Phe-OCH_3 7 and its dimer analog 2.

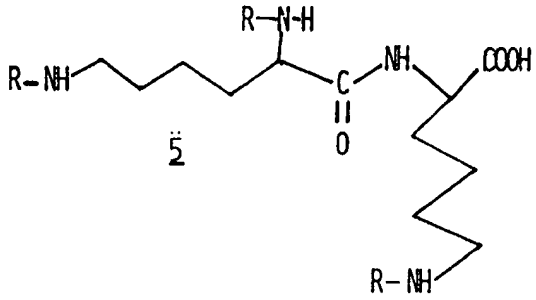
SCHEME 1



1 R = -PHE-LEU-MET-NH-C(=O)-H

2 R = -PHE-LEU-PHE-TBOC

3 R = -H



5

4 R = -PHE-LEU-MET-NH-C(=O)-H

TBOC- PHE -LEU-PHE-OCH_3 7

H-C(=O)-NH-MET-LEU-PHE-OCH_3 6

TABLE I

Comparison between the PD50's of the different species studied. The ED50's of some chemotactic peptides are also included.

	Biological Results		Permeation Results	
COMPOUNDS	ED50 ($Mol.L^{-1}$) Lysosyme release	Relative Potency %	PD50 ($Mol.L^{-1}$)	Relative Potency %
6	100×10^{-9}	100	4.5×10^{-3}	100
1	2.0×10^{-9}	5000	6.3×10^{-5}	7000
4	3.0×10^{-10}	33000	6.0×10^{-5}	7500
3	no activity	-	8.1×10^{-4}	550
5	no activity	-	4.15×10^{-4}	1100
7	antagonist	-	2×10^{-3}	100
2*	antagonist	-	6.5×10^{-5}	3100

* The relative potency of 2 was calculated with respect to that of 7

R = -PHE-LEU-MET-NH-C(H)=O

R = -PHE-LEU-PHE-TBOC

R = -H

4 R = -PHE-LEU-MET-NH-C(H)=O

H-MET-LEU-PHE-OCH_3 6

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Permeation Results	
PD50 ($Mol.L^{-1}$)	Relative Potency %
4.5×10^{-3}	100
6.3×10^{-5}	7000
6.0×10^{-5}	7500
8.1×10^{-4}	550
4.15×10^{-4}	1100
2×10^{-3}	100
6.5×10^{-5}	3100

spect to that of 7

Their PD50's were found to be 2.0×10^{-3} $M.L^{-1}$ and 6.5×10^{-5} $M.L^{-1}$ respectively. This is in line with what one would have expected from the previous discussion.

The use of synthetic phospholipid such as DHP in the preparation of bilayered model membrane in place of natural phospholipids is probably not the ideal model of membrane, since the normal phospholipid components of the cell surface present on their outer layer all the possible chemical groups required to establish the interaction with the exogenous peptides but the permeation results show discrepances which are in line with the lysosomal enzyme activity. Of course the intensity of the biological responses ED50 and PD50 cannot be directly and quantitatively correlated since polypharmacophoric compounds 4 (ED50 33000 at 3.10^{-10}M) and compound 1 (ED 50 5000 at 2.10^{-9}M) have pratically the same permeation activity but in comparison with the corresponding monomeric compound 6 the PD 50 values are significantly different almost two order magnitude.

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L13          537 S L11 AND ( DIME? OR TRIM?)
L14          138 S L11 AND (  TRIM?)
L15           37 S L11 AND (  TRIMER?)
L16         1258 S DOPAMINE (3A) TRANSPOR? (3A) INHIBI?
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L20           14 S L16 AND (DIMER OR DIMERIC OR BIVALENT OR DIVALENT OR LINKER)
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L22           45 S L16 AND (LINKED)
L23           31 DUP REM L22 (14 DUPLICATES REMOVED)

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